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13. ABSTRACT (Maximum 200 words)

   The goal of this research project is to develop a biologically relevant method for fractionating aqueous metals into toxic (bioavailable) and non-toxic forms. The overall approach is: (1) to determine the binding affinity of the gills of fish and other aquatic animals for specific metals using a novel competition bioassay technique; (2) to operationally modify the performance characteristics of cation exchange chromatography to match the metal binding affinity for gill tissue; and (3) to validate and, as necessary, calibrate the cation exchange chromatography method so as to match the toxicity of the metal(s) to aquatic biota under differing water quality conditions. Progress to date includes compiling a library of copper-organic acid stability constants, and completing experiments designed to establish experimental conditions for copper-fish-organic acid competition bioassays. The ILL (incipient lethal level) of copper for rainbow trout was estimated, and the effect of calcium exposure and calcium acclimation concentration on copper toxicity was evaluated.

14. SUBJECT TERMS
water quality, metals, copper, fish

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The overall goal of this research project is to develop a biologically relevant method for fractionating aqueous metals into toxic (bioavailable) and non-toxic forms. The theoretical and procedural bases of the research project are modifications of our earlier work with aluminum (Fernandez 1992, Kline 1992). Research during the first two years of the current AFOSR project emphasizes copper with applications to other metals to be addressed later.

The overall approach is: (1) to determine the binding affinity of the gills of fish and other aquatic animals for specific metals using a novel competition bioassay technique; (2) to operationally modify the performance characteristics of cation exchange chromatography to match the metal binding affinity for gill tissue; and (3) to validate and, as necessary, calibrate the cation exchange chromatography method so as to match the toxicity of the metal(s) to aquatic biota under differing water quality conditions.

(a) Objectives of Research

The specific research objectives of this AFOSR project, as we have subdivided and/or expanded them from our proposal, are as follows:

(1) Compile a library of copper-organic acid stability constants from the published geochemical literature for a series of organic acids that could be used in copper-fish-organic acid competition bioassays.

(2) *Establish experimental conditions to be used in copper-fish-organic acid competition bioassays.*

(3) Determine fish gill-copper binding affinity using competition bioassays.

(4) Modify cation exchange chromatography procedures to match the binding affinity of fish gills for copper.

(5) Evaluate the performance of the cation exchange chromatography procedure in determining the bioavailable (toxic) fraction of copper for other aquatic biota and for differing water quality conditions.

(6) Complete objectives 1 through 5, above, for one or more other metal(s).

(b) Status of Research Effort

During the first year of research we have essentially completed work on objectives 1 and 2, above, and have initiated work on objectives 3 and 4. Our progress has been summarized in the sub-sections below, numbered to correspond with the objectives listed above.

(1)   Compile Copper-Organic Acid Stability Constants

To establish appropriate water quality conditions for the competition bioassays and to select organic acid ligands with a range of copper binding affinities, copper speciation was calculated for a number of water quality conditions and a series of organic·acid ligands. The calculations were performed on a Microsoft Excel spreadsheet. The basic approach is to compile a list of all metal species of interest and to establish their relationships using stability constants from the literature (e.g., Sillen and Martell 1964, Smith and Martell 1974-1982). Mass balance restrictions then yield a nonlinear system of equations which are solved in an iterative calculation cycle, using some "best guess" starting value.

The spreadsheet currently allows one to calculate the interactions between two metals and two ligands. A typical calculation could involve copper, calcium (relevant because it is present at much higher concentrations than copper in our competition bioassays), the major organic acid ligand aimed at complexing copper, and another ligand (chloride, sulfate, etc.) potentially interfering with the copper complexes of interest. Also included are metal-hydroxy complexes, carbonate complexes, and an activity correction (extended Debye-Huckel).

Examples of the results of these calculations for four different organic acid ligands (with two acids at two different acid/copper ratios) are summarized in Table 1 for pH 6 and in Figures 1, 2 and 3 for a pH range of 5 to 7. It is evident from these tabulations and plots that only relatively strong ligands (e.g., as strong as or stronger than citric acid with a copper-acid stability constant of 7.17) will bind copper strongly enough to substantially reduce free copper concentrations. It is also evident from Figures 1, 2 and 3 that the binding behavior is pH-dependent and the optimum pH for binding copper is slightly different for each organic acid, but they are all near optimum around pH 6. Calcium typically cannot compete with copper for these organic acid ligands, although calcium concentrations are about 1000 times higher than total copper concentrations in these calculations and in the competition bioassays to be conducted during this project. The relative amount of calcium bound to the organic ligand is higher for ligands that only contain oxygen functional groups (e.g., citric acid), as opposed to ligands where there are also nitrogen groups available (e.g., NTA, 2,6-pyridinedicarboxylic acid).

In the future more acids will be included in the calculations, the only restriction being that stability constants for their copper complexes must be available in the published literature. The calculation of free copper and the distribution of copper and the organic ligand over the various species will help in interpreting the results from our competition bioassays, and possibly in finding the organic acid that most closely matches the copper binding affinity of the fish gill.

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Table 1. Complex formation between copper and four organic acid ligands with copper at 5 ppb, calcium at 5 ppm, pH 6 and with organic acid:copper ratios of 5:1 and 1:1. See Figures 1, 2 and 3 for plotted concentrations of inorganic copper and copper complexes at pH values from 5 through 7.

Complex Formation between Copper and Selected Ligands

total Cu	5 ppb
total Ligand	in excess of copper, as indicated
total Ca	5 ppm
other solutes:	NaCl, ≈2 ppm
pH	6

	Malonic Acid	Citric Acid	2,6-Pyridine-dicarbox. A.	2,6-Pyridine-dicarbox. A.	NTA	NTA
excess acid:	5x	5x	1x	5x	1x	5x
log K	5.7	7.17	9.94	9.94	14.37	14.37
highest pK	5.696	6.396	5.07	5.07	10.334	10.334
inorg. Cu 1)	4.51	3.33	1.07	0.05	0.20	0.00
Cu(OH)n 2)	0.12	0.09	0.03	0.00	0.01	0.00
CuCO3	0.02	0.01	0.00	0.00	0.00	0.00
Cu bound 3)	0.49	1.67	3.93	4.95	4.80	5.00
Ca bound 3)	0.25	8.82	0.59	11.16	0.02	2.17
free Lig.	39.50	28.28	0.09	1.77	0.50	49.83
all concentrations as ppb						

Expressed as % of total Cu, Ca, or Acid

	Malonic Acid	Citric Acid	2,6-Pyridine-dicarbox. A.	2,6-Pyridine-dicarbox. A.	NTA	NTA
inorg. Cu 1)	90.3 %	66.6 %	21.3 %	1.0 %	4.0 %	0.0 %
Cu(OH)n 2)	2.4 %	1.8 %	0.6 %	0.0 %	0.1 %	0.0 %
CuCO3	0.3 %	0.2 %	0.1 %	0.0 %	0.0 %	0.0 %
Cu bound 3)	9.7 %	33.4 %	78.7 %	99.0 %	96.0 %	100.0 %
Ca bound 3)	0.0 %	0.2 %	0.0 %	0.2 %	0.0 %	0.0 %
free Lig.	96.5 %	37.4 %	0.7 %	2.7 %	3.3 %	66.3 %

1) sum of Cu2+, all hydroxy species, and carbonate complex
2) sum of hydroxy species
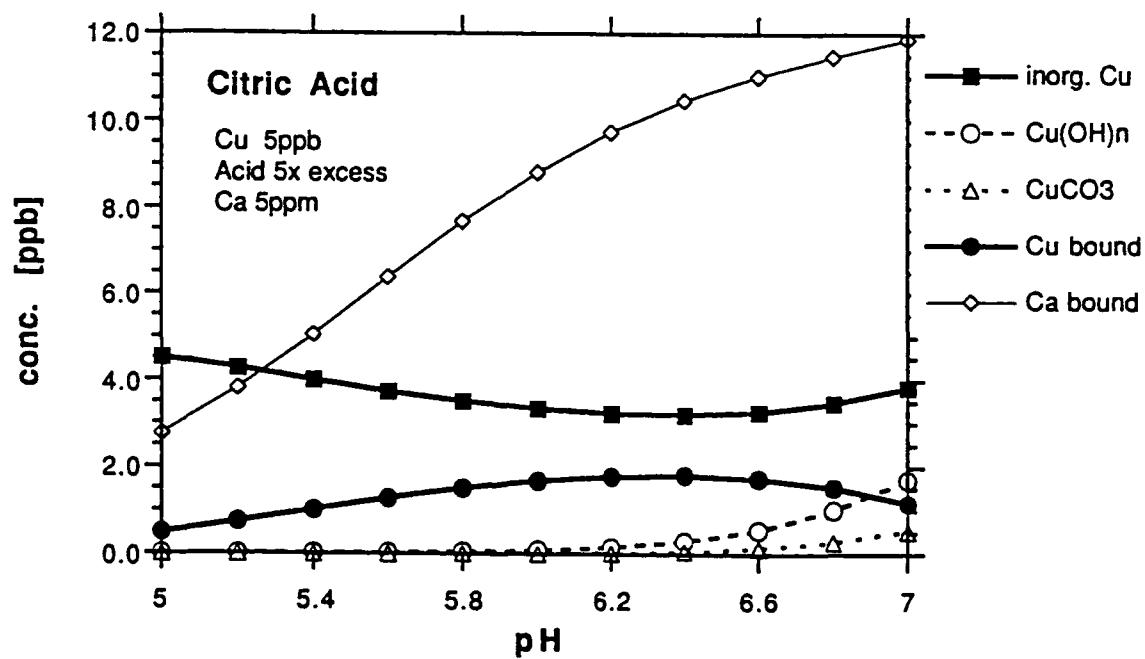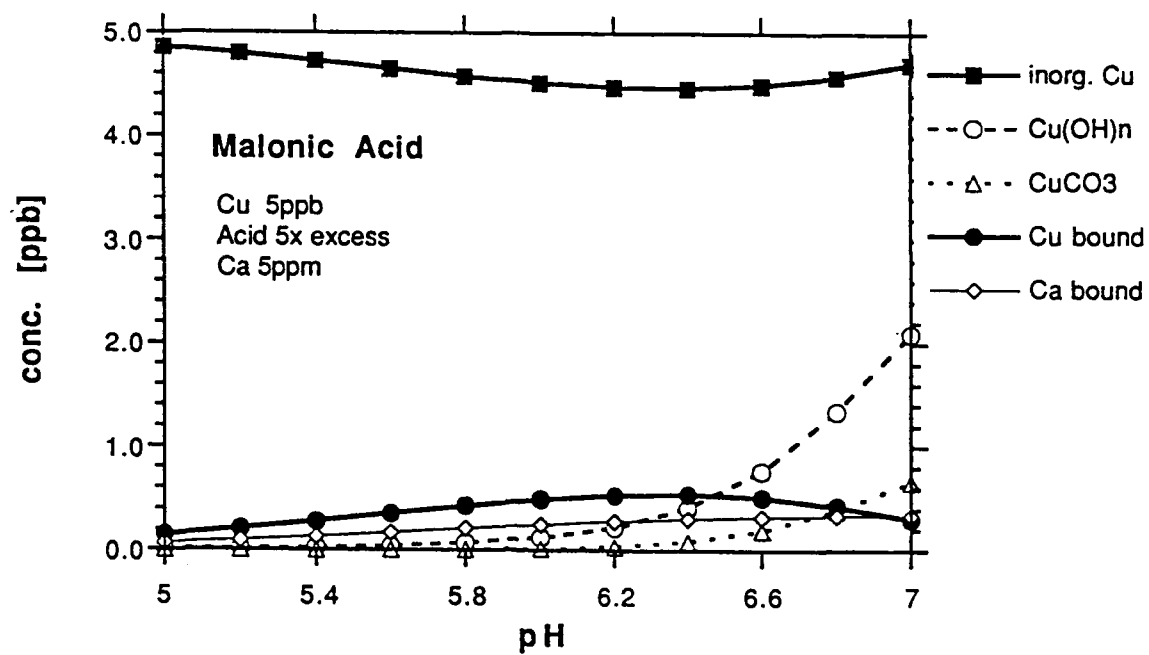3) sum of Cu/Ca bound to acid

Figure 1.   Copper complexation by malonic and citric acids with organic
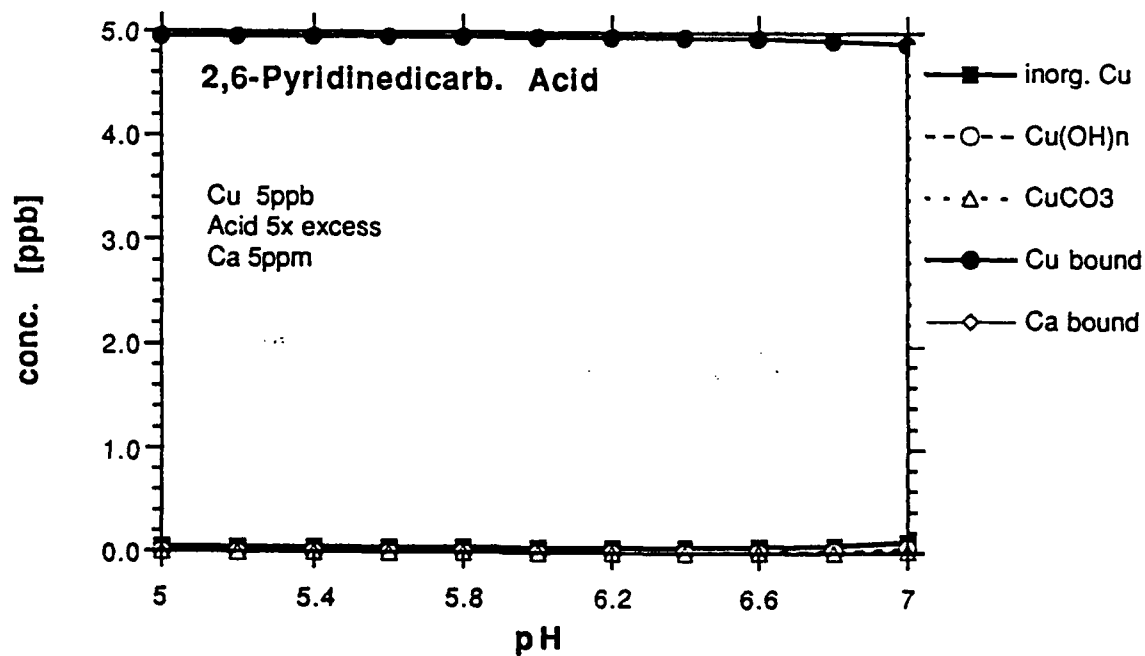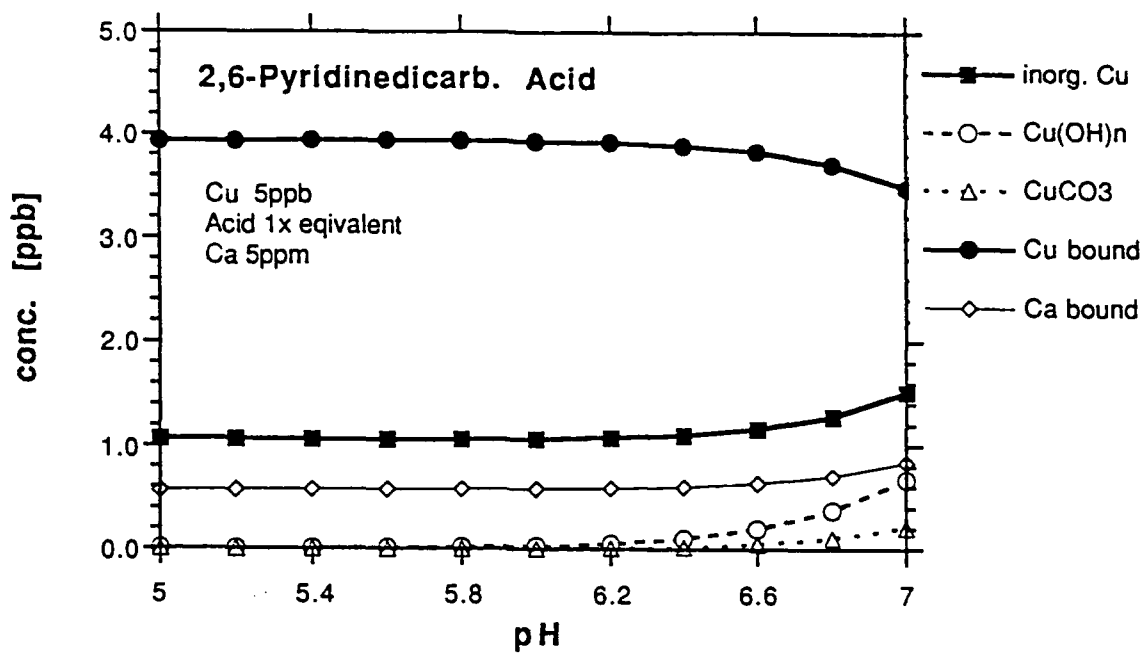acid:copper ratios of 5:1 and at pH 5 through 7.

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Figure 2.   Copper complexation by 2,6-pyridinedicarboxylic acid with organic acid:copper ratios of 1:1 (upper panel) and 5:1 (lower panel) and at pH 5 through 7.
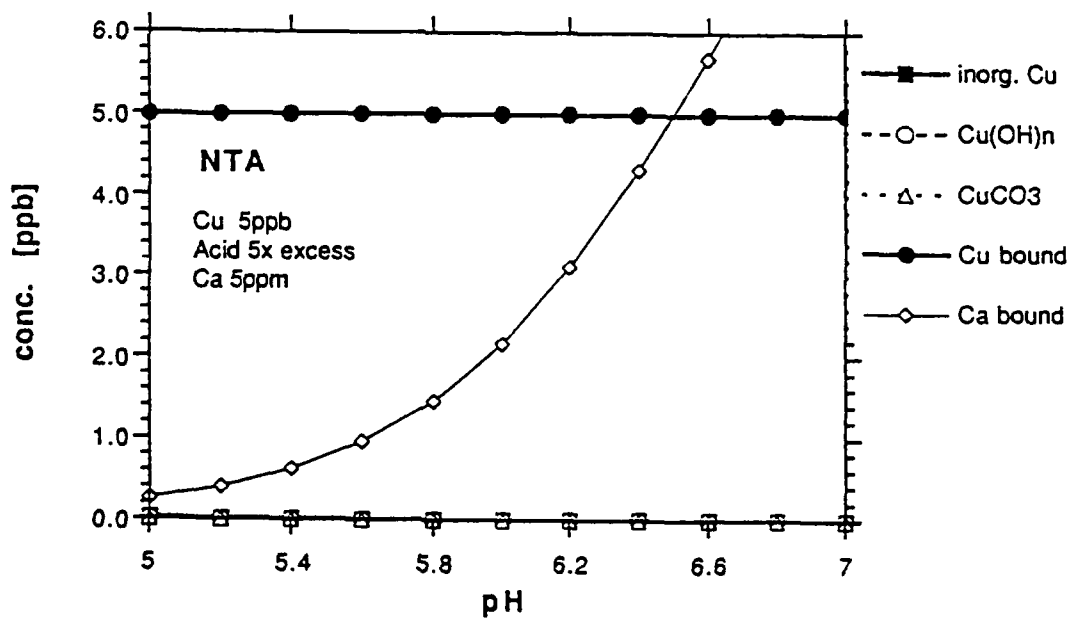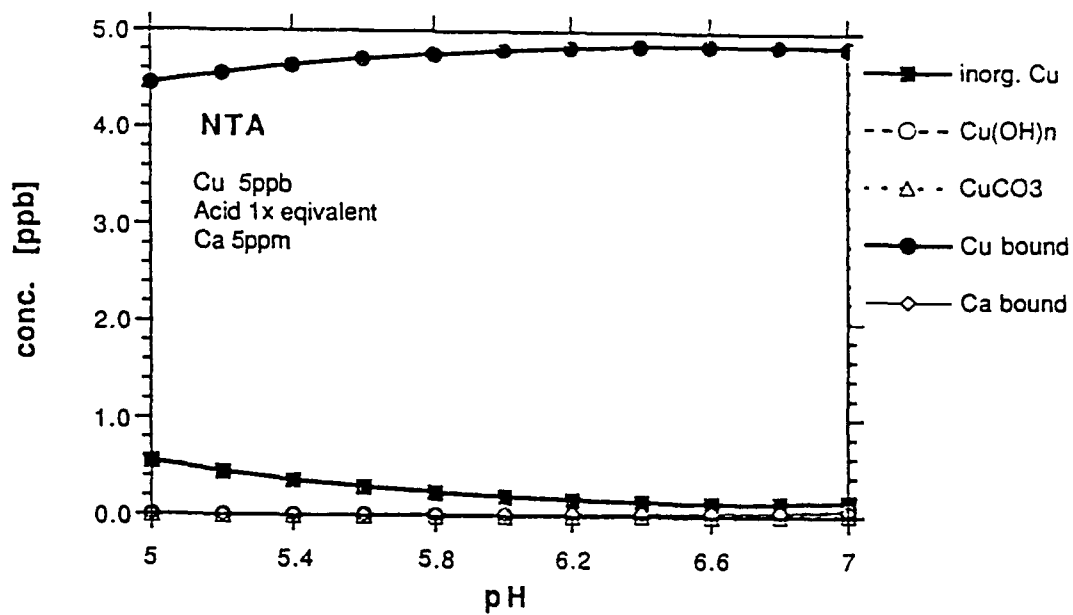
Figure 3. Copper complexation by NTA with organic acid:copper ratios of 1:1 (upper panel) and 5:1 (lower panel) and at pH 5 through 7.

(2)   Establish Experimental Conditions for Competition Bioassays

Prior to determining the copper binding affinity of fish gills using copper-fish-organic acid competition bioassays, it was first necessary to optimize experimental conditions, particularly with respect to copper and calcium concentrations to be used in the bioassays. Appropriately toxic copper concentrations had to be selected so that organic acids with different copper binding affinities (strong affinity and protective to fish versus weak affinity and not protective) could be compared on the basis of fish mortality. Also, since calcium can compete with copper for binding sites on fish gills as well as for binding sites on organic acids, it was necessary to select an optimal calcium concentration so that calculation of metal complexation and speciation could be as straightforward as possible, at least for the first sets of competition bioassays. The discussion below summarizes the experiments we have completed to establish the experimental conditions to be used in the copper-fish-organic acid competition bioassays.

(2.1)   Copper incipient lethal level (ILL)

Three sets of experiments were performed to determine the ILL (incipient lethal level or time-independent LC_{50}) of copper for rainbow trout (Oncorynchus mykiss). The current USEPA water quality criteria document for copper and available published literature on copper toxicity all report LC_{50} values for fish based on relatively high calcium concentrations (e.g., water hardness values of 30 ppm as $CaCO_3$, or higher). However, for the competition bioassays under objective 3, above, to be most successful, it would be favorable to expose fish to mixtures of copper and organic acids at very low calcium concentrations so that calcium does not out-compete copper for the organic acid ligand. Therefore, we determined the ILL for copper at a calcium concentration of 1 ppm. From the results shown in Figure 4, the ILL under these conditions was calculated to be 6.9 ppb copper, and the time to the ILL was 20.6 hours.

Ideally, the lower curve component in Figure 4 (defined by the regression equation $LC_{50} = 8.53 - 0.08 * Time$) should have zero slope, which would result in the regression line intersecting the ordinate to yield a truly time-independent LC_{50}. To achieve this optimal determination of time-independent LC_{50} (ILL), it will be necessary to conduct a fourth experiment using a lower set of copper concentrations and a longer exposure period.

(2.2)   Effect of calcium exposure and calcium acclimation
              concentration on copper toxicity

Two sets of experiments were performed to determine the effect of small differences in calcium concentration and the effect of different levels of calcium acclimation on copper toxicity. As mentioned earlier, the ameliorating effects of calcium on heavy metal toxicity have been well documented. And, in fact, hardness is the only water quality parameter used by the USEPA as a modifier in calculating
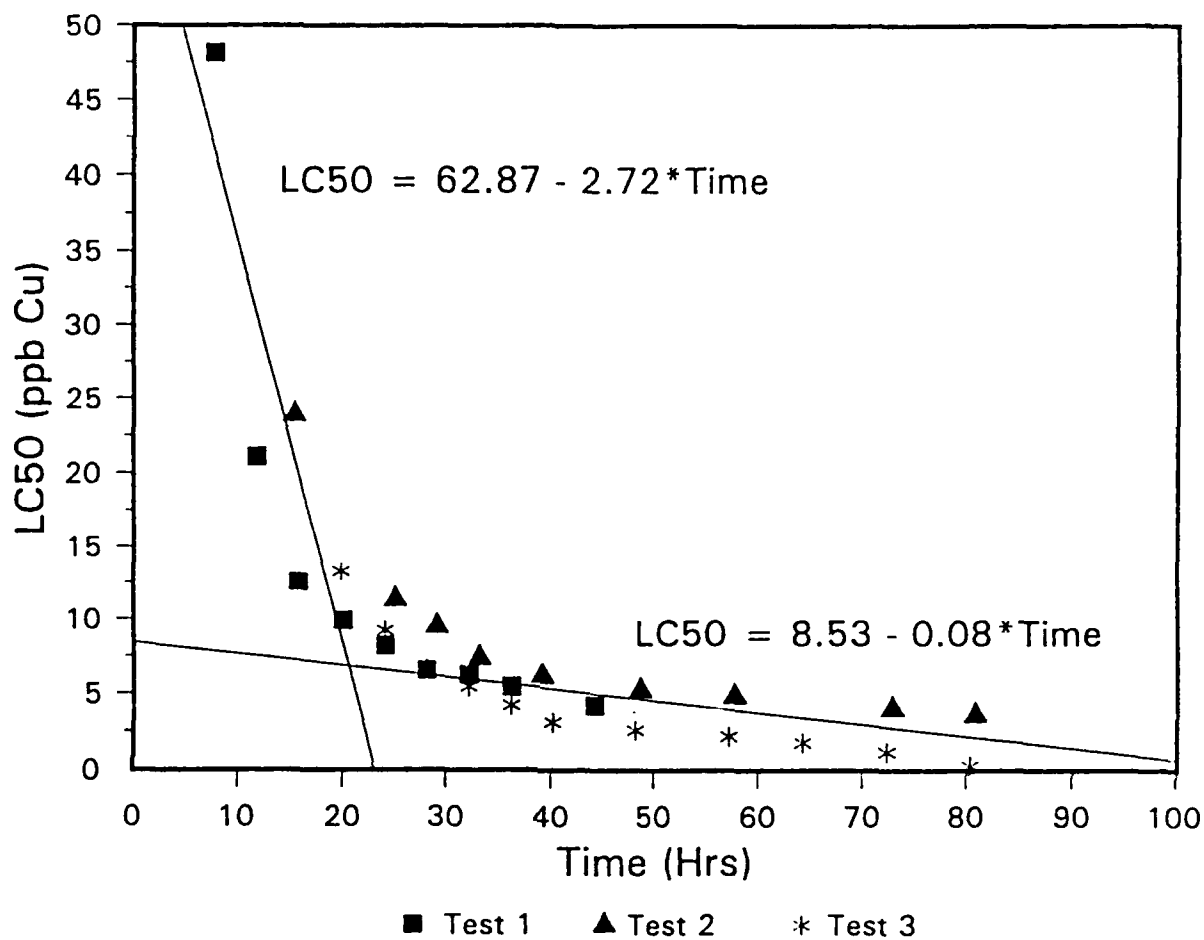
Figure 4. Determination of the copper Incipient Lethal Level (ILL) with data combined from three experiments conducted at 1 ppm calcium and pH 6. ILL estimate equals 6.9 ppb copper at 20.6 hours as determined by the intersection of linear regressions on the two curve components.

permissible concentrations of metals in surface waters. In spite of this, there is very little data available to estimate the influence of small differences in calcium concentration (particularly at very low calcium concentrations in the range of 1 to 10 ppm) on copper toxicity. Moreover, even less data are available to determine the effect of acclimation to different calcium concentrations on copper toxicity. Thus, to select the optimum calcium concentration for fish acclimation and for conducting the competition bioassays, it was necessary to conduct the two sets of experiments described below.

In the calcium exposure experiment, rainbow trout were acclimated for five months to 1 ppm calcium and then placed in 3 ppb copper at each of five different calcium concentrations (1, 2.5, 3.5, 6.5, and 10.5 ppm calcium). The results plotted as percent survival versus time (Figure 5, upper panel) clearly show a marked protective effect of calcium on copper toxicity. This protective effect of calcium is also demonstrated with the results presented as percent survival at 96 hours and as the LT_{50} (the time to 50 percent mortality), as shown in Figure 6 (cross-hatched bars in both upper and lower panels). It is also clear from Figure 5 (upper panel) and Figure 6 (cross-hatched bars) that increased calcium concentration from 1 though 3.5 ppm produced stepwise increases in survival, and that 6.5 and 10.5 ppm calcium completely protected rainbow trout from the lethal effects of 3 ppb copper under these experimental conditions.

In the experiment to determine effects of different calcium acclimation concentrations on copper toxicity, rainbow trout were acclimated to treatment calcium concentrations (1, 2.5, 3.5, 6.5, and 10.5 ppm calcium) for fourteen days prior to the same exposure regimen used in the previous experiment (3 ppb copper at each of the 5 calcium concentrations). The results, this time, were markedly different than when fish were all acclimated to only 1 ppm calcium. As is evident from Figure 5 (lower panel) and Figure 6 (solid bars), there was no consistent difference in fish mortality in the different calcium acclimation/exposure treatment concentrations. And, remarkably, the fish from higher calcium acclimation concentrations (>1 ppm calcium) were more sensitive to copper toxicity than were fish acclimated to only 1 ppm calcium, when both groups were tested at identical calcium concentrations.

Comparison of the results from these two sets of experiments leads to the initial conclusion that the results are counter-intuitive. We would have expected that fish acclimated to 1 ppm calcium and exposed to copper at a higher calcium concentration (e.g., 10.5 ppm calcium) would exhibit higher mortality than fish acclimated to 10.5 ppm calcium and exposed to copper at 10.5 ppm calcium. But, in fact, the opposite response was consistently observed in these experiments.

There are a number of possible explanations for these results. For instance, we know from previous research in our laboratory and from the published literature that very low calcium concentrations (0.5 to 1 ppm) stimulate a number of gill structural changes including an increase in the number and total volume density of mucous cells and, presumably, this could lead to an increase in mucus secretion when the fish are

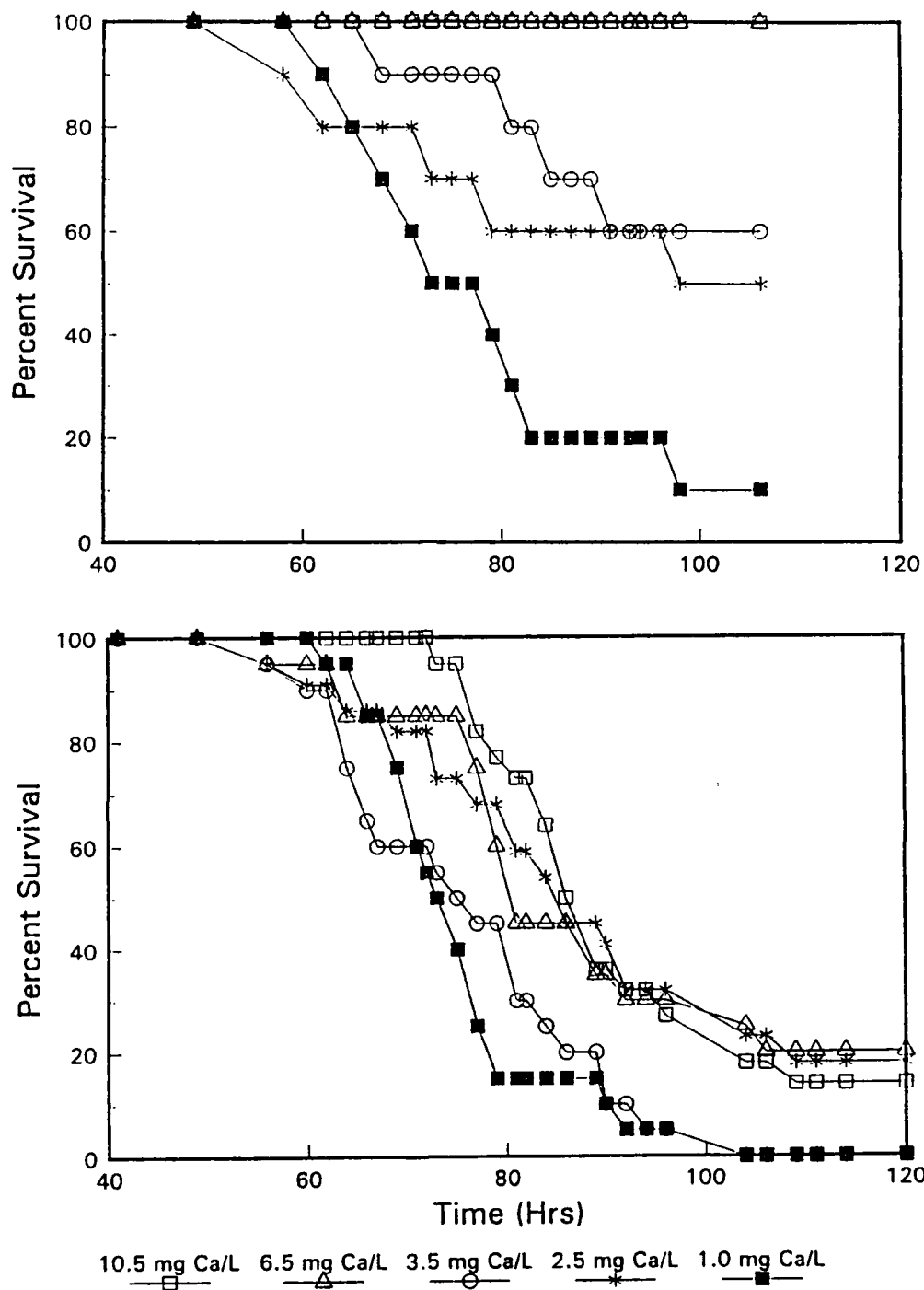Figure 5. Survival plots for rainbow trout exposed to 3 ppb copper and a series of calcium concentrations from 1.0 to 10.5 ppm. The top panel illustrates results with fish acclimated to 1.0 ppm calcium for 5 months; the bottom panel illustrates results with fish acclimated to exposure calcium concentrations (i.e., 1.0, 2.5, 3.5, 6.5 or 10.5 ppm) for 14 days prior to copper/calcium exposure.

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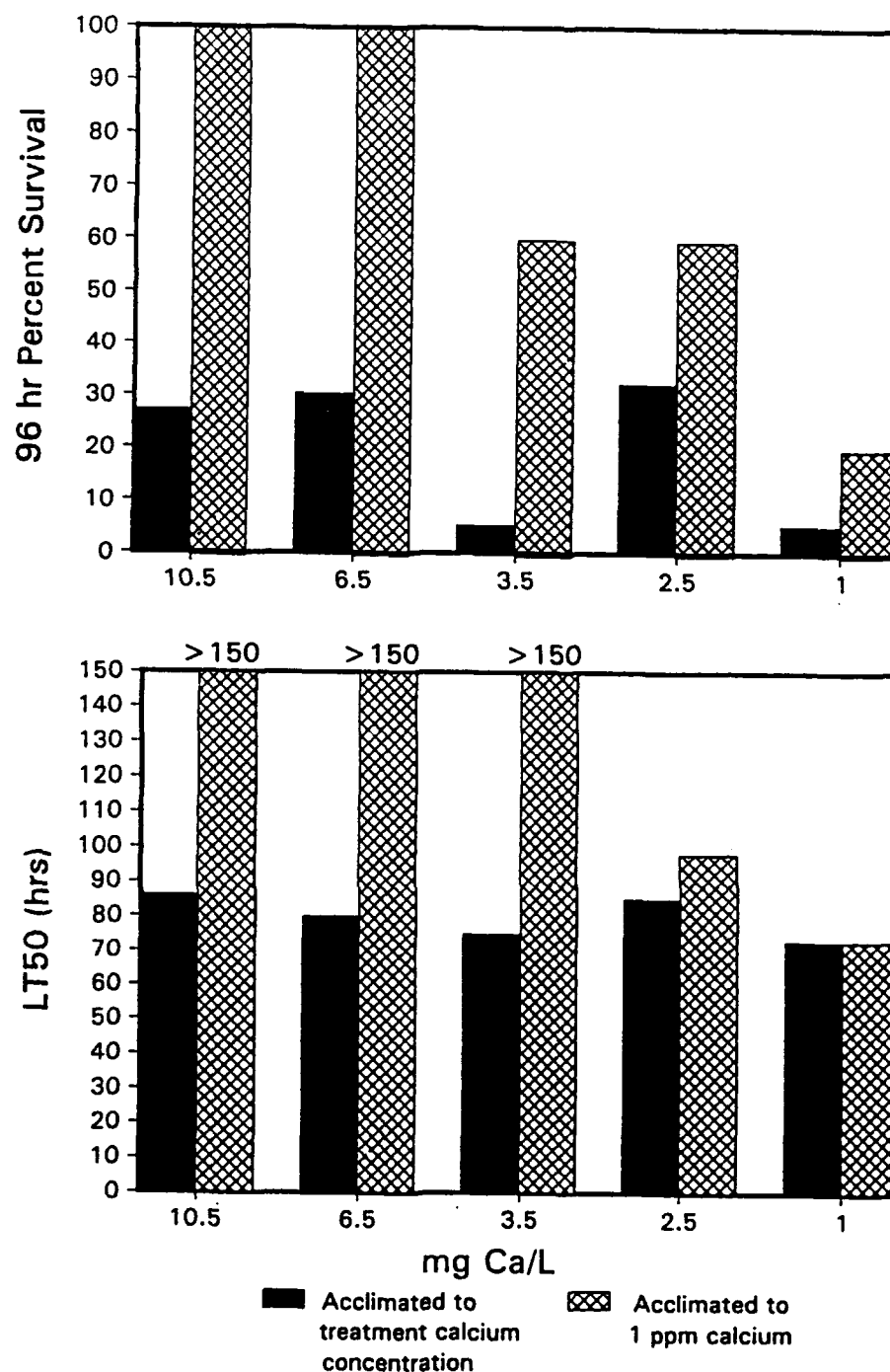Figure 6. Survival/mortality responses of rainbow trout exposed to 3 ppb copper at a series of calcium concentrations, with fish either acclimated to exposure treatment calcium levels for 14 days (solid bars) or acclimated to 1 ppm calcium (cross-hatched bars) prior to copper exposure. The top panel illustrates percent survival at 96 hours; the bottom panel illustrates time to 50 percent mortality (LT50).

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subsequently exposed to copper. It is very possible that an interaction between variable amounts of mucus secretion (as affected by acclimation concentration of calcium) and competitive copper-calcium binding on the gill will need to be understood ultimately to fully explain the results of these two sets of experiments. But since we collected no data on gill structural changes or physiological performance of these fish, especially as they relate to ionoregulatory or respiratory mechanisms of copper toxicity, we are unable to offer definitive explanations of the result at this time.

In spite of the above discussion, the practical consequences of the results from the calcium exposure and acclimation experiments, as they apply to the copper-fish-organic acid competition bioassays, are straightforward. From these experiments we can conclude that the competition bioassays should be conducted as follows: (1) calcium concentrations for the acclimation and exposure periods should be identical to simplify interpretation of fish mortality responses to copper exposure; and (2) the calcium concentration selected for these experiments should be 3 to 5 ppm for a copper exposure concentration of 3 to 5 ppb.

(3)   Determine Fish Gill-Copper Binding Affinity

All preparations for the copper-fish-organic acid competition bioassays are now complete, and fish are being acclimated prior to initiating the experimental exposures. Completed preparations have included the following:

- Constructed and tested the water delivery and copper diluter systems required for the competition bioassays;

- Upgraded our effluent treatment system for reliable 100% removal of metals from our wastewater stream;

- Selected water quality conditions for acclimation and exposure of rainbow trout in the competition bioassays, specifically acclimation for a minimum of 14 days at 1 ppm sodium, 5 ppm calcium and pH 6, and exposure to 5 ppb copper and the same sodium, calcium and pH levels as used for acclimation;

- Selected organic acids to be used in the first series of competition bioassays, specifically citric acid and 2,6-pyridinedicarboxylic acid.

(c)   **Written Publications in Technical Journals, etc.**

Bergman, H.L. Improving on Empiricism: The Potential Value of a Mechanistic Understanding of Bioavailability in Regulating Water Quality. Proceedings of the 1992 SETAC Pellston Aquatic Toxicology Workshop, August, 1992. (In Preparation).

(d)   Professional Personnel Associated with Research

- Harold L. Bergman, Professor, Principal Investigator
- James I. Drever, Professor, Co-Principal Investigator
- Joseph D. Fernandez, Research Associate
- David D. Gulley, Research Associate
- Norbert G. Swoboda-Colberg, Research Associate
- Russell K. MacRae, Graduate Research Assistant (M.S.)
- Darren E. Smith, Graduate Research Assistant (M.S.)

(e)   Interactions

(i)   Papers Presented at Meetings, etc.

Bergman, H.L. Physiological Mechanisms of Metal Toxicity to Fish. Annual Meeting of the Western Division, American Fisheries Society, Fort Collins, Colorado. July, 1992. (Abstract Submitted).

MacRae, R.K., D.E. Smith, and H.L. Bergman. Apparent Cu Binding Affinity for Rainbow Trout Gills. Annual Meeting of the Society of Environmental Toxicology and Chemistry, Cincinnati, Ohio. November, 1992. (Abstract Submitted).

(ii)   Consultative and Advisory Functions to DoD

None

(f)   New Discoveries, Inventions, etc.

None

(g)   Other Information

As this project was just getting started, Joseph Fernandez, a CoPI and full time Research Associate on the project, accepted a career position with the U.S. EPA Environmental Research Laboratory in Duluth, Minnesota. We have since recruited two new graduate students and obtained additional technical assistance from Norbert Swoboda-Colberg and other Research Associates who had been working on other projects. These personnel changes resulted in a slower than anticipated start, but now that the project team has been restructured we expect to be back on our originally planned schedule by fall 1992.

(h)   References

Fernandez, J.D. 1992 (expected). A methodology for determining bioavailable aluminum in acidic surface waters. Ph.D. Dissertation, University of Wyoming, Laramie.

Kline, E. 1992. The effects of organic complexation on aluminum toxicity to rainbow trout (Oncorhynchus mykiss). M.S. Thesis, University of Wyoming, Laramie.

Sillen, L.G. and A.E. Martell. 1964. Stability Constants of Metal-Ion Complexes. Special Publication No. 17. The Chemical Society. London, England. 754 p.

Smith, R.M. and A.E. Martell. 1974-1982. Critical Stability Constants. Vols. 1-5. Plenum Press, New York, NY.